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Matrix metalloproteinase-2 is downregulated in sciatic nerve by streptozotocin induced diabetes and/or treatment with minocycline: Implications for nerve regeneration

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ABSTRACT

Minocycline is an inhibitor of matrix metalloproteinases (MMPs) and has been shown to have analgesic effects. Whilst increased expression of MMPs is associated with neuropathic pain, MMPs also play crucial roles in Wallerian degeneration and nerve regeneration. In this study we examined the expression of MMP-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1/-2 in the sciatic nerve of control and streptozotocin-induced diabetic rats treated with either vehicle or minocycline by quantitative PCR and gelatin zymography. We assessed the effects of minocycline on nerve conduction velocity and intraepidermal nerve fibre (IENF) deficits in diabetic neuropathy and investigated the effects of minocycline or MMP-2 on neurite outgrowth from primary cultures of dissociated adult rat sensory neurons.

We show that MMP-2 is expressed constitutively in the sciatic nerve *in vivo* and treatment with minocycline or diabetes leads to downregulation of MMP-2 expression and activity. The functional consequence of this is IENF deficits in minocycline-treated nondiabetic rats and an unsupportive microenvironment for regeneration in diabetes. Minocycline reduces levels of MMP-2 mRNA and nerve growth factor-induced neurite outgrowth. Furthermore, *in vivo* minocycline treatment reduces preconditioning-induced *in vitro* neurite outgrowth following a sciatic nerve crush. In contrast, the addition of active MMP-2 facilitates neurite outgrowth in the absence of neurotrophic support and pre-treatment of diabetic sciatic nerve substrata with active MMP-2 promotes a permissive environment for neurite outgrowth. In conclusion we suggest that MMP-2 downregulation may contribute to the regenerative deficits in diabetes. Minocycline treatment also downregulates MMP-2 activity and is associated with inhibitory effects on sensory neurons. Thus, caution should be exhibited with its use as the balance between beneficial and detrimental outcomes may be critical in assessing the benefits of using minocycline to treat diabetic neuropathy.

Introduction

Diabetic neuropathy (DPN) is a common secondary complication of diabetes mellitus, which is associated with biochemical and structural changes in the nervous system including nerve conduction velocity (NCV) deficits and altered mechanical and thermal sensitivity (Tomlinson and Gardiner, 2008). A common feature of both clinical and experimental diabetic neuropathy is degeneration of distal nerve fibres and a reduced capacity for regeneration of injured axons (Beiswenger et al., 2008; Christianson et al., 2006; Kennedy and Zochodne, 2000; Kennedy and Zochodne, 2005). This leads to numbness, loss of protective sensation and an increased risk of amputation. Other than glycaemic control there is no effective treatment and current strategies which treat neuropathic pain do not encourage neuronal repair (Tesfaye et al., 2013). There is therefore an unmet clinical need for effective treatments for DPN targeting both nerve regeneration and neuropathic pain.

Minocycline is a semi-synthetic analogue of tetracycline and a widely prescribed generic drug used to treat microbial infections. It has been shown to have anti-inflammatory, neuroprotective, anti-oxidative, and/or analgesic effects in a number of disease models and this paired with the good safety record of minocycline in patients has paved the way for clinical studies in a number of neurodegenerative diseases (Plane et al., 2010). The mechanisms underlying the neuroprotective effects of minocycline are unclear since minocycline inhibits multiple targets including matrix metalloproteinase (MMP)-2 and MMP-9. The upregulation of MMPs has been linked to the development and maintenance of neuropathic pain (Kawasaki et al., 2008). In experimental diabetic neuropathy the analgesic potential of minocycline has been explored (Bhatt and Veeranjaneyulu, 2010; Morgado et al., 2011), but none of these studies have investigated the effects on regeneration.

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MMPs are a large family of zinc-dependent proteolytic enzymes, best known for their role in tissue remodelling, degradation of extracellular matrix (ECM) molecules, release of ECM-sequestered growth factors and cleavage and activation of membrane-bound cytokines and their receptors (Sternlicht and Werb, 2001). Expression and activity of MMPs are tightly regulated at three levels: by transcriptional regulation; by activation — they are secreted in an enzymatically inactive state as pro-enzymes which are activated in a proteolytic manner by direct cleavage of the pro-peptide by another MMP or a non-proteolytic manner by organomercurials; and by inhibition *via* interactions with tissue inhibitors of MMPs (TIMPs) (Sternlicht and Werb, 2001).

In the peripheral nervous system the coordinated expression of ECM, MMPs and TIMPs during Wallerian degeneration and nerve regeneration governs the success of nerve repair (Gantus et al., 2006). Following nerve injury, there are spatial and temporal alterations in the expression of MMPs. MMP-9 is rapidly and transiently upregulated at sites of peripheral nerve injury (Demestre et al., 1999; Ferguson and Muir, 2000; Shubayev and Myers, 2000) with increased expression in Schwann cells, blood vessels, and activated macrophages. MMP-9 facilitates blood nerve barrier breakdown enabling macrophage and neutrophil infiltration (Siebert et al., 2001) and is essential to Wallerian degeneration (Hughes et al., 2002; Ide, 1996; Johnson et al., 2005; Mantuano et al., 2008). MMP-2 mRNA and protein levels increase later and remain upregulated up to 63 days post-nerve crush. Early MMP-2 expression is in Schwann cells and endoneurial cells (Demestre et al., 2004; La et al., 1996; Muir, 1994) whilst later MMP-2 is increased within the regenerating axons and degrades inhibitory ECM components promoting a permissive environment for regeneration (Ferguson and Muir, 2000; Zuo et al., 1998). MMP-2 knockout mice show reduced regeneration of axons following spinal contusion injury and impaired functional recovery (Hsu et al., 2006) highlighting the importance of MMPs for successful regeneration.

A number of studies have described an increase in systemic MMPs in diabetes (Jacqueminet et al., 2006) and altered expression of MMPs has been implicated in the pathophysiology of a number of secondary complications including nephropathy (Thrailkill et al., 2009) and poor wound healing (Liu et al., 2009). To date, there is little information about the expression or activity of MMPs and/or their role in the regenerative deficits associated with diabetic neuropathy. The aims of this study were (1) to compare the expression of MMP-2, MMP-9 and TIMP-1/2 in the peripheral nerve of control rats and rats with streptozotocin (STZ)-induced diabetes; (2) to assess the effects of minocycline on indices of experimental diabetic neuropathy and regulation of MMPs in the peripheral nerve; and (3) to investigate the effect of MMP-2 and minocycline on *in vitro* sensory neuron growth.

Materials and methods

Reagents

All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

Animal studies

All animal studies and procedures were licensed under the UK Animals (Scientific Procedures) Act 1986. Diabetes was induced in adult male Wistar rats (250–325 g, Charles River, UK) with freshly dissolved STZ (55 mg/kg in sterile saline, i.p.), administered following an overnight fast. Diabetes was verified 3 days post-STZ (OptimumPlus; MediSense, UK) and rats with blood glucose >15 mmol/l were classified as diabetic. STZ-diabetic and age-matched control rats were maintained for 4–12 weeks. For the minocycline-prevention study: rats were randomly allocated into one of four treatment groups (control-vehicle ($n = 8$); control-minocycline ($n = 8$); diabetic-vehicle ($n = 10$); diabetic-minocycline ($n = 10$)). Minocycline was administered by daily gavage rather than the intraperitoneal route to avoid deposition of minocycline in the peritoneal cavity, which has been associated with inflammatory lesions and variability in absorption (Fagan et al., 2004). Treatment with minocycline 25 mg/kg, p.o or vehicle (water, p.o.) commenced 3 days post-STZ, and continued daily for the duration of the trial. Animals were monitored daily, weighed twice weekly and maintained for 8 weeks post-STZ. Body weight and blood glucose data are shown in Table 1.

An additional group of adult male Wistar rats (250–300 g) was daily dosed with either minocycline (25 mg/kg p.o, $n = 4$) or vehicle (water, p.o, $n = 4$) for 3 days. Rats were then anaesthetized with isoflurane (2% in oxygen) and, under sterile conditions, the left sciatic nerve was exposed at mid-thigh level. The sciatic nerve was crushed with the tips of watchmaker's forceps (2 × 15 s) the wound was closed in layers and the animals recovered under observation. Dosing continued daily for a further 3 days, then rats were killed and ipsilateral and contralateral lumbar (L)4/5 dorsal root ganglia (DRG) were removed for cell culture and sciatic nerves removed for PCR 3 h post last dose of minocycline.

Nerve conduction velocity (NCV)

One day following the last dose of minocycline, rats were terminally anaesthetized with isoflurane (2–4% in oxygen) and electromyograms were recorded from plantar foot muscles on a Powerlab 4 with ABI Scope software in response to stimulation (1.5–5 V, 2 ms pulses,

Table 1
Diabetic rats are significantly lighter and hyperglycaemic compared to age-matched nondiabetic control rats. Minocycline had no significant effect on body weight or terminal blood glucose levels of control or diabetic rats (note, 1/10 rats from the diabetic-vehicle group and 5/10 rats from the diabetic-minocycline group became normoglycaemic between 4 and 8 weeks post-STZ — their data was excluded from all analysis). For blood glucose levels greater than the upper limit of detection of glucose meter, data was assigned as 27.8 mmol/l. Data represents mean ± s.d. ***p < 0.001 *t*-test and two-way ANOVA followed by Bonferroni posthoc tests.

Duration (weeks)	Animal group (*n* numbers)	Start body weight (g)	End body weight (g)	End blood glucose (mmol/l)
Timecourse study				
4	Age-matched control (10)	340 ± 9	471 ± 7	8.1 ± 1
	Diabetic (12)	339 ± 7	370 ± 14***	27.8 ± 0***
8	Age-matched control (10)	335 ± 5	579 ± 18	9.1 ± 1
	Diabetic (12)	343 ± 3	383 ± 10***	27.8 ± 0***
12	Age-matched control (10)	334 ± 6	612 ± 14	9.9 ± 1
	Diabetic (12)	342 ± 5	396 ± 11***	27.8 ± 0.0***
Minocycline study:				
8	Nondiabetic-vehicle (8)	358 ± 20	580 ± 39	5.3 ± 0.8
	Nondiabetic-minocycline (7)	364 ± 20	567 ± 63	6.8 ± 2.3
	Diabetic-vehicle (9)	366 ± 19	383 ± 50***	27.7 ± 0.0***
	Diabetic-minocycline (5)	365 ± 12	431 ± 81***	27.5 ± 0.2***